Cutaneous Melanoma: Predictors of Patient Survival and the Potential of Priming of the Sentinel Lymph Node

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2011

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Chapter 9

Summarizing Discussion and Future Prospects

Summarizing Discussion

As reviewed in **Chapter 1**, complete surgical excision at an early stage remains the only curative treatment for cutaneous melanoma with few available adjuvant therapy options. Nevertheless, melanoma is a relatively immunogenic tumor type and particularly amenable to immunotherapeutic approaches. (1) A dense network of cutaneous Dendritic Cells (DC) may account for the reported efficacy of vaccination through the skin and provides an attractive target for the immunotherapy of melanoma. Several phenotypically distinct DC subsets are discernable in the skin: a.o. epidermal Langerhans Cells (LC) and dermal DC (dDC). Upon appropriate activation both subsets can efficiently migrate to melanoma-draining lymph nodes (LN) to prime T cell mediated responses. (2) Unfortunately, from an early stage, melanoma development is characterized by strong immune suppression, facilitating unchecked tumor growth and spread. Particularly the primary tumor site and the first-line tumor-draining LN, the so-called Sentinel LN (SLN), bear the brunt of this melanoma-induced immune suppression -and these are exactly the sites where anti-melanoma effector T cell responses should be primed by DC in order to prevent early metastasis. (3-5) Through local immunopotentiation the dermis may be utilized as a portal to activate DC and kick-start or boost effective T cell-mediated anti-melanoma immunity, even in the face of this immune suppression.

Clinical observations in melanoma patients

In **Chapter 2** we investigated both the influence of the diagnostic biopsy type and the presence of residual tumor cells in the re-excision specimen on disease free and overall survival. After (partial) removal of a pigmented skin lesion 471 patients were diagnosed with stage I/II melanoma and underwent re-excision and a sentinel node biopsy. All patients were followed prospectively for up to 5 years. Patients were divided according to their diagnostic biopsy type (wide excision biopsy (n=279), narrow excision biopsy (109), excision biopsy with positive margins (n=52) and incisional biopsy (n=31)) and the presence of residual tumor cells in their re-excision specimen (n=41). Survival analysis was done using Cox's proportional hazard model adjusted for eight important confounders of melanoma patient survival. Neither the diagnostic biopsy type nor the presence of tumor cells in the re-excision specimen influenced disease-free and overall survival of melanoma patients. Nevertheless, routine use of incision biopsies is not recommended. Incisional biopsies often consist of only a small percentage of the surface area of the pigmented skin lesion, making it difficult to sample a representative area within the tumor. (6) Furthermore, when melanoma is diagnosed, attempting to evaluate the depth of invasion in an incisional biopsy is treacherous and may lead to over- or underestimation of the invasion. (7, 8) Of course these problems are less prominent in excision biopsies with positive margins, where the majority of the lesion has been removed and only the outer borders are compromised making a sampling error highly unlikely. With melanoma incidence rates rising (9) and early detection of melanoma still being the only way to improve melanoma patient survival, (10) it is important for all physicians to feel confident about removing a pigmented skin lesion suspect for melanoma. Incisional biopsies are not recommended but there is no cause for concern when an excision biopsy turns out to have positive margins.

In **Chapter 3** we evaluated the clinical outcome of stage I/II melanoma patients who underwent selective SLN dissection after a follow-up (FU) of at least 10 years. We analyzed several covariates of long-term patient survival, including the influence of the number of additional positive lymph nodes at complete lymph node dissection (CLND) and the sentinel lymph node tumor burden on melanoma patient survival. To this end, 224 clinically stage I/II cutaneous malignant melanoma patients with a FU of 10 years were analysed. Forty-three patients were SLN positive and information concerning the size of the LN metastasis was available for all patients. Disease free and overall

survival analyses were performed using the Kaplan-Meier approach. Cox's proportional hazards regression model was used to analyze DFS and OS associated covariates. The overall and disease-free survival rates continued to decrease after 5 years. This was most marked for patients with additional positive lymph nodes (ALN) at CLND, and patients with a SLN tumor load of >1.0 mm^2. We recommend that SLN procedures be performed for melanomas of ≥0.9 mm Breslow and that all patients with a tumor of less than 1 mm Breslow should not need to be included in follow-up, regardless of their SLN status. The first 5 years remain the most critical period for follow-up, especially for patients with a positive SLN, ulceration, lymphatic invasion and certainly patients with positive ALN at CLND. Nonetheless, we feel that high risk patients should receive special attention and careful monitoring for at least a decade.

Immunohistochemistry of primary tumors and SLN from stage I/II melanoma patients

The cellular immune system plays an important role in the immune response in melanoma patients. (11, 12) In **Chapters 4 and 5** we looked at the influence of tumor infiltrating lymphocytes (TILs) on SLN status and clinical outcome. In **Chapter 4** we investigated whether presence of TILs and Natural Killer (NK) cells correlated with expression of MHC-I or MHC-II on primary tumor cells and/or antigen presenting cells and if this is was related to clinical outcome in clinically stage II melanoma patients. Diagnostic primary melanoma samples from 35 melanoma patients were examined and evaluated for the presence of granzyme B^+ (GrB), $CD8^+$, $CD4^+$ and/or $CD56^+$ TIL populations after immunohistochemical staining. Furthermore, expression of MHC-I and -II on tumor and/or tumor infiltrating cells was examined. Expression of PI-9, a GrB inhibitor, in melanoma cells was also determined and correlated to survival. Progression-free survival time was taken as endpoint for survival analysis. Presence of activated cytotoxic TILs was found to predict favourable prognosis independent from gender, Breslow thickness, ulceration and sentinel node status. Moreover, presence of activated cytotoxic TILs was significantly correlated with expression of MHC-I on tumour cells and with expression of MHC-II on intra-tumoral antigen presenting cells. The presence of activated cytotoxic TILs and putatively intact antigen presentation in the context of MHC-I and MHC-II in primary melanomas predicted a highly favourable outcome in clinically stage II melanoma patients. These data strongly support the notion that an intact cellular immune response is a major factor in preventing (early) melanoma cell invasion and dissemination.

In **Chapter 5** the possible association between the presence of activated and/or suppressive TILs and SLN status in clinically stage I/II melanoma patients was explored. Diagnostic primary melanoma samples from 20 patients with an SLN metastasis were compared to melanoma samples from 20 patients with a negative SLN. The patients were matched for gender, age and Breslow thickness. Presence of activated GrB^+ TILs, of suppressive ($FoxP3^+$) TILs and of MHC class I antigen expression on tumor cells were analysed by immunohistochemistry. FoxP3 and MHC-I expression had no direct bearing on the presence of melanoma metastases in the SLN. Whereas the presence of activated GrB^+ TILs in the primary melanoma had no predictive value for SLN status either, their absence was strongly associated with the presence of metastasis in the SLN. While both GrB^+ and $FoxP3^+$ TILs could be detected in SLN metastases, a majority of these metastases did not display MHC-I expression. These data support a role for cytotoxic T cells in the prevention of early metastasis of melanoma to the draining lymph nodes and suggest the presence of suppressive $FoxP3^+$ TILs to be responsible for melanoma escape and spread in the presence of activated effector T cells. These results are in accordance with previous studies demonstrating that the presence of lymphocytes in melanoma biopsies is associated with a favourable clinical outcome. (13-15) Moreover, our data demonstrate that melanoma infiltrating T-lymphocytes consist of $CD4^+$, and activated $CD8^+$ and

GrB$^+$ cytotoxic T-lymphocytes populations. Activation of these cytotoxic T-lymphocytes depends on specific MHC-I restricted antigen recognition and on the presence of co-stimulatory expressing and cytokine producing CD4$^+$ T helper cells. (16, 17) Because we observed a strong correlation between the presence of TILs and intact MHC-I expression on melanoma cells, our data support the idea that loss of MHC-I expression results in failure to mount a cellular anti-melanoma response. Therefore, loss of MHC-I is as such a possible powerful immune escape mechanism. (18, 19) The importance of a cellular response in the primary tumor can also be seen in the fact that absence of GrB$^+$ TILs in primary melanoma biopsies is strongly associated with the presence of SLN metastasis. These data underscore the notion that an activated cellular immune response is important in preventing melanoma cells to disseminate to lymph nodes.

Priming of the SLN of melanoma patients with GM-CSF and/or PF-3512676 CpG-B

Impaired immune effector functions in the melanoma SLN may allow for early metastatic events. Local administration of PF-3512676 (formerly known as CpG 7909) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) have shown immunostimulatory effects of both DC and T-cell subsets in preceding studies. (20-27) Previously, we carried out two small single-blinded phase II studies assessing the effects of GM-CSF or CpG in melanoma patients. In the first study 12 stage I melanoma patients were included to receive four daily intradermal (i.d.) injections of GM-CSF (at 3 μg/kg) or saline. (28, 29) We monitored the phenotype of DC in the SLN through flowcytometry and assessed the frequency and functionality of CD8$^+$ T cells responding to specific melanoma-derived peptides in an IFNγ-Elispot assay. The GM-CSF-receiving patients showed a significant increase in the number and maturation state of CD1a$^+$ conventional DC (cDC), which was associated with a more robust melanoma-specific CD8$^+$ T cell response in the SLN, as compared to saline injected patients. Also, melanoma-specific T cell rates correlated directly to mature CD83$^+$CD1a$^+$ DC frequencies in the SLN, confirming the importance of properly activated DC in the induction of an anti-melanoma immune response. In the second phase II study 23 stage I/II melanoma patients received one i.d. injection of either 8 mg PF-3512676 or saline. (30) PF-3512676 administration resulted in bulkier SLN and higher yields of isolated SLN leukocytes. Also, a clear enhancement of both plasmacytoid DC (pDC) and CD1a$^+$ cDC maturation and activation and a significant decrease in Treg frequencies was seen in the SLN. In addition, PF-3512676 administration was associated with the presence of a newly identified CD11c^{hi}CD123$^+$CD83$^+$TRAIL$^+$ mature SLN-cDC subset and an increased release of a variety of inflammatory cytokines.

In **Chapter 6** we set out to ascertain whether these PF-3512676-induced immunostimulatory effects translated into higher frequencies of melanoma-specific CD8$^+$ T cells. CD8$^+$ T cells from SLN and peripheral blood were tested for reactivity by IFN-γ ELISPOT assay against several HLA-A1/A2/A3-restricted epitopes derived from various Melanoma-Associated Antigens (MAA) in 21 of 24 enrolled patients. Frequencies of NK cells and frequencies and maturation state of DC subsets in the SLN were determined by flow cytometry. Melanoma-specific CD8$^+$ T-cell response rates against more than one MAA-derived epitope in the SLN were 0 out of 11 for the saline group versus 5 out of 10 for the PF-3512676-administered group. Of these 5 responding patients, 4 also had a measurable response to more than one MAA epitope in blood. Furthermore, a clear relationship was found between increased frequencies in the SLN of both MAA-specific CD8$^+$ T cells or NK cells and CpG-induced pDC maturation. These data show an increase in melanoma-specific CD8$^+$ T-cell frequencies as well as an increased effector NK cell rate after a single dose of PF-3512676. In addition, the significant correlation between activated SLN-pDC on the one hand and NK cells and melanoma-specific CD8$^+$ T cell reactivity in both the SLN and the post-treatment blood samples on

the other hand, strongly suggests local and systemic protection against tumor spread to specifically result from direct PF-3512676-induced pDC activation rather than from indirect (e.g. IFNα-meditated) cDC activation. Altogether these data suggest a role for local PF-3512676 administration as adjuvant treatment in early-stage melanoma in combatting metastatic spread.

In **Chapter 7** we assessed the combined effects of low-dose GM-CSF and PF-3512676 CpG-B on cDC and pDC subsets in the SLN, in an effort to strengthen the immune defence against metastatic spread. As we observed predominant effects of GM-CSF on $CD1a^+$ cDC and of the CpG-B compound on pDC and non-classical $CD1a^-CD11c^{hi}$ cDC, we aimed for full-range DC subset activation by combining both. To ascertain the added value of GM-CSF to the use of CpG monotherapy, we conducted a 3-arm phase II study in which 28 stage I-III melanoma patients were randomized to receive i.d. injections around the primary tumor excision site of saline or low-dose CpG-B (1 mg PF-3512676), alone or combined with low-dose GM-CSF (100 µg), 7 and 2 days prior to excision of the SLN. SLN cells and PBMC were analyzed by flow cytometry. Significantly increased maturation of all identifiable cDC and pDC subsets was observed in the SLN upon administration of combined CpG/GM-CSF, as well as recruitment of two $CD1a^-CD11c^{hi}$ cDC subsets (one $CD14^-$ and the other $CD14^+$). The latter both expressed BDCA3/CD141, suggestive of cross-priming ability. (31) Activation of cDC, pDC and monocytes was also observed in peripheral blood. Correlative and *in vitro* analyses indicated CpG-B and GM-CSF to activate and recruit BDCA3/$CD141^+$ cDC from the blood to the SLN. We conclude that combined CpG/GM-CSF delivery at the primary melanoma excision site results in a more powerful and wide-ranging DC subset activation in SLN and blood, than achieved by CpG alone. The concerted activation of pDC and cDC subsets as well as recruitment of BDCA3/$CD141^+$ cDC subsets with putative cross-priming ability will further strengthen protective anti-melanoma immunity.

In most preclinical models, and in some clinical studies, $CD8^+$ cytotoxic T cells have been recognized as the main effectors mediating tumor regression (32-36), and they are therefore the focus of most immune monitoring schemes. (37-39) Despite recent advances, accurate monitoring of vaccine- and/or tumor-specific T cell responses remains difficult, due to extremely low frequencies of tumor-specific T cells at effector sites (40-43), necessitating their polyclonal expansion prior to functional testing. (44) Several protocols are available for polyclonal T cell expansion, but they are not necessarily interchangeable in terms of the subset or differentiation state predominance of the expanded T cells. In **Chapter 8** we therefore set out to compare a novel 4-1BBL-artificial Antigen-Presenting Cell (aAPC) based method (45-47) to classic CD3/CD28 antibody-based methods (48, 49) for the expansion of functional MAA-specific $CD8^+$ effector T cells from clinical LN samples.

SLN were obtained from early-stage melanoma patients participating in our clinical phase II trial, receiving intradermal low-dose CpG-B ODN PF-3512676 (see Chapter 7) or saline. T cells from SLN samples were expanded using plate-bound anti-CD3/CD28 antibodies, anti-CD3/CD28-coated beads (both combined with IL-2) or K32/4-1BB aAPC, i.e. irradiated K562 cells transfected with the human low-affinity Fcγ receptor CD32 (loaded with the anti-CD3 OKT3 monoclonal antibody) and the co-stimulatory molecule 4-1BBL (combined with IL-15). Expanded T cells were analyzed for the presence of memory/effector $CD8^+$ T cells and the recognition of, and responsiveness to, common recall antigen- or MAA-derived epitopes by various assays. Our data show that K32/4-1BBL aAPC are superior to plate- and bead-bound anti-CD3/CD28 antibodies for the expansion of functional effector-memory $CD8^+$ T cells from immunomodulated SLN, as demonstrated by combined analysis of tetramer binding, IFNα/CD107a expression and cytokine release. We conclude that 4-1BB-mediated expansion of in vivo primed effector-memory $CD8^+$ T cells by means of aAPC facilitates sensitive monitoring of functional anti-tumor immunity in small samples from tumor-draining LN.

Future Prospects

As seen in Chapter 3, survival of melanoma patients continues to decline in the ten years after diagnosis, especially for patients with thick tumors, a high SLN tumor load and positive ALN. We strongly advise any physician dealing with potential melanomas to avoid incisional biopsies in order to correctly determine the Breslow thickness and stage melanoma patients as stipulated in Chapter 2. Therapeutic options in advanced melanoma are limited and complete surgical excision at an early stage remains the only curative treatment option. Progress has been made in recent years in unravelling the interaction between the tumor and the immune system which has led to interesting new developments in melanoma treatment. The immunogenic characteristics of melanomas provide an opportunity for the immune system to attack and eradicate the tumor cells. TILs play an important role in this line of defence as indicated by our data presented in Chapters 4 and 5. Unfortunately, melanoma development is characterized by strong immune suppression. This early suppression is most marked at the primary tumor site and in the SLN. We believe that, even before distant metastasis occurs, local immunopotentiation of the primary tumor site and its draining LN is a valid strategy to (re-)establish local immune control. The dermis provides an ideal site for delivery of these compounds, as reviewed in Chapter 1. A network of DC and effector-memory T cells in the dermis are poised for re-activation and subsequent generation of an effector immune response. Moreover, the dermis provides ready access to draining LN, where respective memory and naïve T cell responses can be further boosted or primed. Importantly, the dermis-targeted and localized immunomodulatory strategies discussed in Chapters 6 and 7 may not only provide protection against local tumor spread: the activated effector T cells can also recirculate and home to distant metastatic sites (as demonstrated by our findings in Chapter 6). Thus, also patients in later stages of melanoma may experience clinical benefit from these approaches.

Novel therapeutics for local immunopotentiation: TLR-L and cytokines

Novel and powerful immunomodulatory agents with proven clinical activity and/or efficacy are becoming available to combat melanoma-imposed immune suppression on both DC and T cells. In the preface of this thesis we discussed several cytokines and TRL ligands as potential adjuvant therapeutic agents. Many more TLR-L with potential anti-tumor efficacy have yet to be tested in a clinical setting. Some of the novel TLR-L currently being developed include ligands for TLR3, -4, -7, and -8. Polyinosinic-polycytidylic acid (poly I:C) is a dsRNA analog recognized by TLR3 that activates NK cells and DC in vivo via TRIF-dependent pathways. Poly I:C was shown to exert anti-melanoma effects *in vivo*, which in part were attributable to the induction of IFN-producing killer DC. (50) Indeed, Poly I:C combined with CpG, either with an intratumoral plasmid DNA encoding CD40L or DC-tumor fusion hybrids, showed promising results in prolonging survival and lowering tumor burden *in vivo* and enhanced the anti-tumor efficacy of a virus-based vaccine in a murine melanoma model. (51)

Combination therapies of TLR-L and other immune modulators hold great promise. As stimulatory TLR-L and cytokines activate immune effector cells through differential down-stream signalling cascades (see Chapter 1, *Figure 2*), their combined administration (like GM-CSF *and* CpG –see Chapter 7) may have additive, or even synergistic effects. Combined administration of multiple TLR-L can similarly lead to synergistically enhanced release of the pro-inflammatory cytokines IL-6, IL-12 and TNFα and involves signalling through PI-3K, MAPK, STAT and NF-κB. (52) However, it can also lead to enhanced release of suppressive IL-10 and subsequent induction of Tregs. (53-55) This unwanted inhibitory effect may be prevented by combining the TLR-L with inhibition of MAPK p38 activity.

(56) Similarly, CpG-mediated TLR9 activation was shown to be attenuated by STAT3 activation, leading to immune suppression. (57) These suppressive effects were shown to be prevented by conjugating CpG ODN to STAT3 siRNA prior to delivery. (58) These findings argue in favour of combining CpG ODN with newly developed small-molecule JAK2/STAT3 inhibitors. (59)
Recently, GM-CSF has received some bad press. Filipazzi et al reported increased frequencies of a $CD14^{+}HLA\text{-}DR^{lo}$ subset of Myeloid-Derived Suppressor Cells (MDSC) in stage IV melanoma patients upon s.c. vaccination with a HSP-peptide complex combined with GM-CSF. (60) In addition, two groups separately reported that repeated administration of GM-CSF, added as adjuvant to peptide or tumor cell-based vaccines, to stage II to IV melanoma patients over a prolonged period of time, resulted in lower anti-vaccine T cell responses (61) and decreased over-all survival rates. (62) A mouse study accompanying these two reports suggested that these findings might be attributable to GM-CSF-induced increases in Treg rates and/or activation status. (63) Indeed, GM-CSF was previously shown to lead to the production by APC of Milk Fat Globule EGF 8 (MFG-E8), which attenuated the vaccination efficacy of GM-CSF-transduced melanoma cells through induction of Tregs in a mouse model. (64) Single low-dose administration of GM-CSF acting in a strictly localized fashion -as proposed in this thesis for local immunomodulation- may not have the detrimental effects described in the above discussed papers, but this remains to be established. The latter mouse studies do, however, provide a rationale for combining GM-CSF administration with strategies aimed at eliminating Tregs or other T cell-mediated suppressive mechanisms (e.g. anti-CD25-conjugated toxins or anti-CTLA4). (65) Importantly, our own data presented in Chapter 7 suggest that also combined administration with CpG-B may counteract some of these reported disadvantages of GM-CSF: combined local administration in the studied patients did not lead to increased Treg or MDSC frequencies. Indeed, local CpG-B monotherapy induced reduced frequencies of peripheral MDSC.

Novel therapeutics for local immunopotentiation: antibodies
Beside TLR-L and cytokines, novel immunomodulatory monoclonal Antibodies (mAbs) are now entering or have entered the clinical testing phase and may also be useful for local immunopotentiation of the SLN.
The CTL Antigen-4 (CTLA4) and PD-1 receptors represent crucial checkpoints in the control of T cell reactivity. (66) Pre-clinical and clinical studies have clearly indicated enhanced anti-tumor efficacy upon blocking of CTLA4 and strongly support further implementation of anti-CTLA4 in immunotherapeutic approaches to the treatment of melanoma. (67-69) Clinical responses and prolonged survival have been observed upon systemic treatment of melanoma patients with the anti-CTLA4 mAbs ipilimumab or tremelimumab. (70-72) Although sometimes these clinical responses coincided with Th17-associated Autoimmune Breakthrough Events (ABE) that in some cases were quite severe. (73) A single local administration of anti-CTLA4 aimed at conditioning of the primary melanoma site and the SLN in an adjuvant setting should allow for the use of relatively low anti-CTLA4 dosages without excess risk of autoimmune effects. In support of localized low-dose application of anti-CTLA4, Simmons *et al.* recently reported favourable results obtained in the B16 melanoma model with vaccination with GM-CSF- and anti-CTLA4-secreting tumor cells. Equivalent anti-tumor activity to systemic administration of high-dose anti-CTLA4 was observed at significantly lower anti-CTLA4 serum levels and with serological evidence of reduced systemic autoimmunity. (74)
PD-1, like CTLA4, forms a checkpoint for T cell activation. Upon binding of PD-1, T cells become inactivated and enter a reversible state of anergy. (75, 76) PD-1 knock-out mice developed autoimmune glomerulonephritis and arthritis. (77, 78) Its ligand B7-H1 is often expressed on tumors and may thus interfere with activation of tumor-infiltrating T cells. (79-81) A first phase I clinical

trial has been conducted to study the effects of i.v. anti-PD-1 (MDX-1106) administration in 39 patients, including 9 melanoma patients. (82) Results were encouraging with signs of clinical anti-tumor efficacy, $CD8^+$ T cell infiltration at tumor sites and relatively mild autoimmune symptoms. This first clinical trial has now opened the possibility to also test anti-PD-1 as a locally applied immunostimulant.

Beside suppression of T cell reactivity, suppression at the DC level is another possible obstacle in the effective triggering of an anti-melanoma immune response. In numerous preclinical studies CD40-mediated activation of DC was identified as a key event in the generation of long-term $CD8^+$ T cell-mediated immunity. (83-87) CD40 stimulation also results in the reversal of T cell tolerance (88), renders DC resistant to the suppressive effects of IL-10 (89), and releases them from the control of Tregs. (90) Findings from a phase I trial of a single systemic administration of the anti-CD40 mAb CP-870,893 (91) showed objective partial responses in four patients with melanoma (i.e. 27% of enrolled melanoma patients), demonstrating the possible utility of CP-870,893 in the immunotherapy of melanoma.

Concluding remarks: future clinical developments

A clear challenge for the immediate future will be to identify the most powerful combinations of the above listed agents (and many others that were not listed!) to effect optimal DC and T cell activation and facilitate the (re-)activation of anti-melanoma immunity. Rather than testing clinical efficacy of monotherapies in large-scale phase III trials, it might be more prudent to first study combination therapies in multiple small-scale and carefully designed phase II trials with biological read-outs, such as the ones described in this thesis. This would allow for the more rational design of multi-targeted therapies with a better chance of attaining improved clinical efficacy in future randomized phase III trials.

On a final note, exciting new developments in the treatment of melanoma include the use of targeted therapeutics to modulate aberrantly activated signalling cascades in melanoma, e.g. in patients carrying activating b-raf mutations. (92) As many of these signalling cascades also affect immune functions, it will be interesting to see how these new therapeutics influence the patient's immune status and how they may be combined with immunotherapeutic approaches.

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